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Mini-Review

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Different subcellular locations of secretome components of Gram-positive bacteria

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Gram-positive bacteria contain different types of secretion systems for the transport of proteins into or across the cytoplasmic membrane. Recent studies on subcellular localization of specific components of these secretion systems and their substrates have shown that they can be present at various locations in the cell. The translocons of the general Sec secretion system in the rod-shaped bacterium *Bacillus subtilis* have been shown to localize in spirals along the cytoplasmic membrane, whereas the translocons in the coccoid *Streptococcus pyogenes* are located in a microdomain near the septum. In both bacteria the Sec translocons appear to be located near the sites of cell wall synthesis. The Tat secretion system, which is used for the transport of folded proteins, probably localizes in the cytoplasmic membrane and at the cell poles of *B. subtilis*. In *Lactococcus lactis* the ABC transporter dedicated to the transport of a small antimicrobial peptide is distributed throughout the membrane. Possible mechanisms for maintaining the localization of these secretion machineries involve their interaction with proteins of the cytoskeleton or components of the cell wall synthesis machinery, or the presence of lipid subdomains surrounding the transport systems.

Introduction

In Gram-positive bacteria, proteins can be sorted to at least four different destinations: the cytoplasm, the cytoplasmic membrane, the cell wall and the extracellular medium. Since protein synthesis takes place in the cytoplasm, proteins functioning at other locations have to be transported into or across the cytoplasmic membrane. Translocated proteins are usually synthesized as precursor (pre-)proteins with an amino-terminal signal peptide. In general, pre-proteins are first recognized by targeting factors, which assist in transport of the pre-proteins to the membrane, where they are translocated through a proteinaceous channel, after which the signal peptide is removed (Tjalsma *et al.*, 2000). Some exported proteins become attached to the membrane by lipid modification of a cysteine residue in the extreme N-terminus of the mature protein, whereas proteins with transmembrane segments are laterally released from the translocation channel to become embedded in the membrane (Tjalsma *et al.*, 2000). Other exported proteins are selectively immobilized in the cell wall by electrostatic or covalent interactions (Navarre & Schneewind, 1999; Leenhouts *et al.*, 1999; Ton-That *et al.*, 2004).

By far the largest number of translocated and membrane proteins in Gram-positive bacteria are predicted to follow

the general protein secretion (Sec) pathway, which involves SecA, SecY, SecE, SecG and a number of accessory proteins (Tjalsma *et al.*, 2000). During or shortly after translocation, secretory pre-proteins are processed by a type I signal peptidase. Lipoprotein precursors are lipid-modified by the diacylglycerol transferase Lgt, and cleaved by the lipoprotein-specific (type II) signal peptidase Lsp. After translocation, exported proteins are folded by different chaperones such as the membrane-attached HtrA, a protein that also possesses protease activity. The twin-arginine translocation (Tat) pathway translocates folded proteins containing a highly conserved twin-arginine motif in their signal peptide (Tjalsma *et al.*, 2000). A fourth major class of leader peptides is found on ribosomally synthesized bacteriocins and pheromones that are exported by ABC transporters (Wandersman, 1998). These leader peptides lack the typical hydrophobic H-domain of Sec signal sequences (Tjalsma *et al.*, 2000), and are removed from the mature protein by a subunit of the ABC transporter, by specific signal peptidases or by general proteases (Wandersman, 1998). Some pathogenic bacteria contain specialized secretion pathways for export of virulence factors, such as the ESAT-6 system (Pallen, 2002).

The (*in vivo*) spatial analysis of proteins by various microscopic techniques has resulted in the observation that these different secretion machineries are located at different sites in the cytoplasmic membrane. In this review the present knowledge concerning the subcellular location of various protein translocation pathways of Gram-positive bacteria and their substrates is described (see Fig. 1). Finally,

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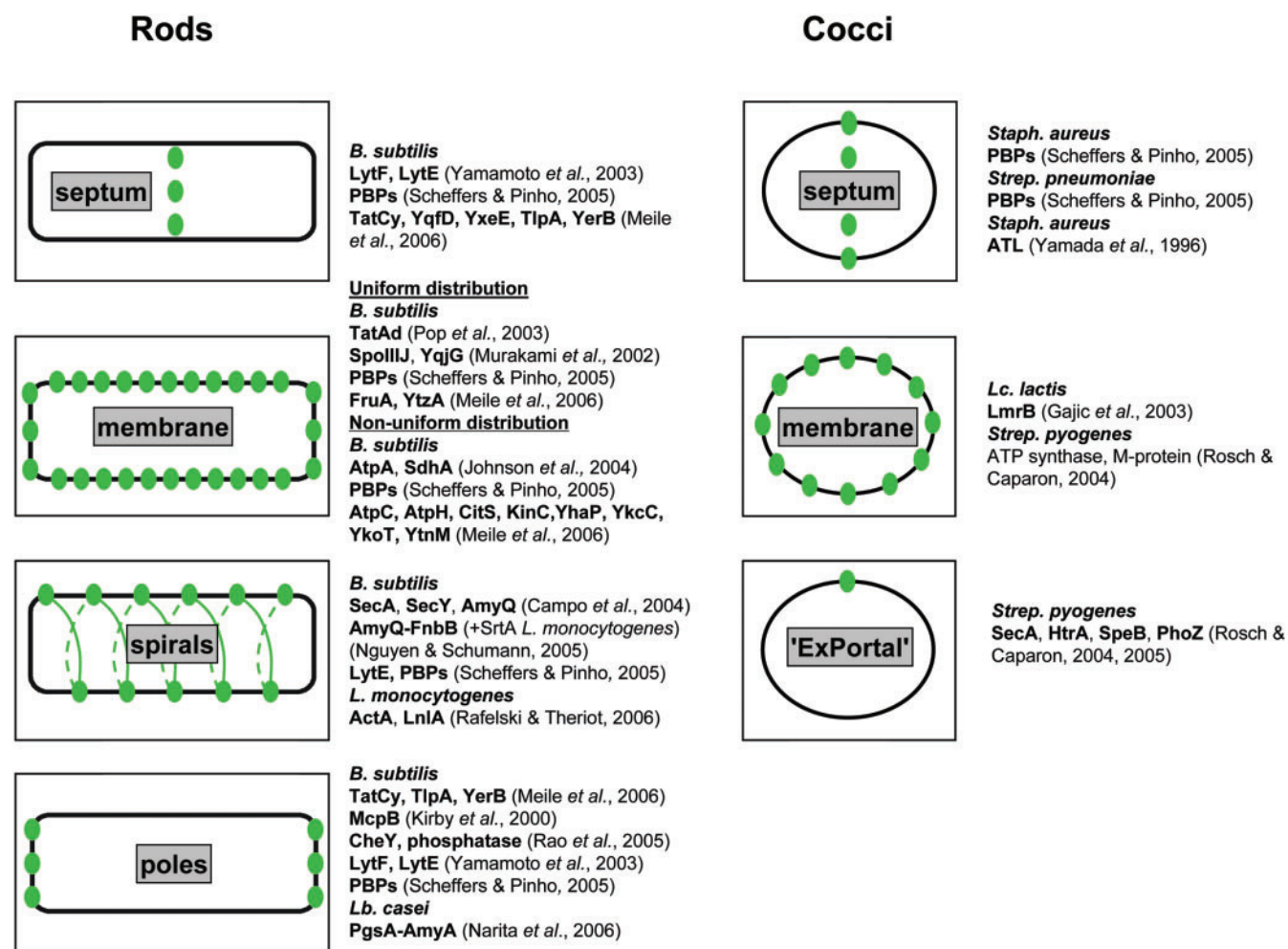


Fig. 1. Graphical representation of the protein localization patterns discussed in this review. See text for details.

possible mechanisms that control this localization are discussed.

Localization of translocation machineries

In the past two years three papers appeared that demonstrated the localization of components of the Sec pathway in the pathogen *Streptococcus pyogenes* and in *Bacillus subtilis*, respectively. Immunogold electron microscopy analysis of thin sections of *Strep. pyogenes*, after reaction with SecA antibodies, showed that a microdomain with a high concentration of Sec translocons called the ExPortal is present in this coccoid bacterium (Rosch & Caparon, 2004). Each streptococcal cell has a single ExPortal, which is positioned at a hemispherical position distal to either cell pole. The secreted cysteine protease SpeB, its maturation protein HtrA and the heterologous alkaline phosphatase PhoZ all co-localize with SecA in this domain (Rosch & Caparon, 2004, 2005). An HtrA derivative that was released into the medium was inefficient in maturing SpeB, indicating that HtrA membrane localization is important

for proper functioning. The ExPortal is proposed to function as an organelle that promotes biogenesis of secreted proteins by coordinating interactions between nascent unfolded secretory proteins and membrane-associated chaperones. In contrast, *B. subtilis* contains multiple sites dedicated to Sec-mediated protein export (Campo *et al.*, 2004). Using GFP fusions, the major components of the Sec machinery, SecA and SecY, were shown to localize at 3 to 10 specific sites per cell near and/or in the cytoplasmic membrane. Immunofluorescence microscopy analysis of localization of SecY and pre-AmyQ, a substrate of the Sec machinery, resulted in a similar pattern. The results suggest that the translocons of *B. subtilis* are organized in spiral-like structures along the cell. This is similar to recent results obtained for *Escherichia coli*, where the Sec machinery is also localized in a helical arrangement (Shiomi *et al.*, 2006).

In mitochondria, chloroplasts and Gram-negative eubacteria, Oxa1p(-like) proteins are critical for membrane protein insertion. *B. subtilis* contains two Oxa1p homologues that are presumably involved in membrane protein

biogenesis and in protein secretion, namely SpoIIIJ and YqjG (Tjalsma *et al.*, 2003). Both proteins are randomly distributed throughout the membrane and are thus not enriched in the vicinity of the Sec machinery (Murakami *et al.*, 2002; Rubio *et al.*, 2005).

In *E. coli*, the localization of components of the Tat pathway has been extensively studied using GFP fusions. These proteins were mostly found throughout the membrane, while in some cases they accumulated at the cell poles (Berthelmann & Brüser, 2004; Ray *et al.*, 2005). In Gram-positive bacteria, localization of components of the Tat pathway has so far only been investigated in *B. subtilis*. A GFP-TatCy fusion protein localizes in the cytoplasmic membrane with foci at the cell poles (Meile *et al.*, 2006). Using immunogold labelling and electron microscopy, TatAd was found to be localized in the membrane as well as in the cytosol, where it can interact with its substrate pre-PhoD (Pop *et al.*, 2003). The membrane-associated protein did not show any clustering or polar preference. However, in the same study the helical localization pattern of SecY was also not apparent (supplementary material in Pop *et al.*, 2003), presumably because only a small fraction of SecY molecules was detected. Apparently, a low density of gold particles can make it difficult to detect certain localization patterns that can be revealed by fluorescence microscopy of GFP-fusion proteins, because in that case every protein is labelled. Therefore, a discrete localization of TatAd can not be excluded, and indeed our recent results suggest that a TatAd-GFP fusion shows enrichment at the cell poles (unpublished results).

The ABC transporter LmrB of *Lactococcus lactis* is a multidrug resistance protein responsible for transport of the bacteriocins LsbA and LsbB (Gajic *et al.*, 2003). A GFP-LmrB fusion protein was distributed all around the cytoplasmic membrane. Whether HtrA, which is responsible for cleavage of the precursor peptide to yield active LsbA, is localized within the vicinity of LmrB remains to be investigated.

Localization of membrane translocated proteins

Integral membrane proteins

Integral membrane proteins can extend into or span the entire bacterial cell wall, exposing parts on the cell surface. GFP fusions with the *B. subtilis* membrane proteins ATP synthase (AtpA) and succinate dehydrogenase (SdhA) were shown to localize to domains rather than being uniformly distributed around the cytoplasmic membrane (Johnson *et al.*, 2004). In another study, two other ATP synthase subunits, namely AtpC and AtpH, as well as the predicted permease YtnM were also shown to be non-uniformly distributed in the membrane (Meile *et al.*, 2006). Both N- and C-terminal GFP fusions with the predicted ABC-type Na⁺ efflux pump protein YhaP are membrane located, with an enrichment seen at the septum. Dual-labelling experiments using AtpA-CFP and SdhA-YFP indicated

partial co-localization in similar submembranous domains. These domains are irregular in shape and have no preference for a specific position. Localization of these protein-containing membrane domains was random and highly dynamic, which leads to the suggestion that integral membrane proteins are free to diffuse around the cytoplasmic membrane, with temporal concentration variations (Johnson *et al.*, 2004). However, there are also integral *B. subtilis* membrane proteins that are uniformly distributed (Meile *et al.*, 2006). In *Strep. pyogenes*, ATP synthase is randomly distributed in the cytoplasmic membrane and thus does not remain at the ExPortal after its membrane insertion (Rosch & Caparon, 2005).

The C-terminus of the membrane protein phosphatidylglycerophosphate synthase (PgsA) of *B. subtilis* is exposed on the cell surface. Fusion of α -amylase (AmyA) of *Streptococcus bovis*, labelled with a FLAG peptide tag, to the C-terminus of PgsA resulted in display of AmyA on the cell surface of the rod-shaped bacterium *Lactobacillus casei* (Narita *et al.*, 2006). The PgsA-AmyA-FLAG fusion protein was shown by immunofluorescence microscopy to localize around the septa of cells. This result might indicate that the C-terminal part of PgsA is translocated at the septum, the site of cell wall synthesis (see below), or that PgsA accumulates at this location. The authors argued that, due to the existence of a smaller number of cell wall components in this part of the cell, it might be more suited for the accumulation of large proteins. ActA of the Gram-positive intracellular bacterial pathogen *Listeria monocytogenes* also spans the bacterial membrane and the peptidoglycan, thereby exposing its N-terminus on the surface (Rafelski & Theriot, 2006). Polar distribution of ActA is required for bacterial actin-based motility and successful infection. Rafelski & Theriot (2006) showed that upon induced expression an ActA-RFP (red fluorescent protein) fusion initially appeared at one to four sites along the cylindrical body of the bacteria, which were distinct from the sites of cell wall synthesis. ActA was thereafter redistributed over the entire cylindrical cell body through helical cell wall growth and accumulated at the poles. No polar secretion was observed: cell wall growth was required and two to three generations were needed for full polar localization. A similar redistribution was obtained for the covalently cell-wall-bound protein LnlA (Rafelski & Theriot, 2006). The authors propose that both proteins gradually accumulate at the hemispherical pole through helical incorporation of cell wall material during several bacterial generations. They also suggest that the Sec translocons must be fixed in space while the cell wall grows around them.

During vegetative growth of *B. subtilis* three main localization patterns have been observed for the integral membrane penicillin-binding proteins (PBPs) (Scheffers & Pinho, 2005). Some of the PBPs show a disperse localization within the membrane, while others specifically localize at the site of cell division. A third group of PBPs appears as distinct spots at the cell periphery, which sometimes resolve in short

arcs. One of the four PBPs of *Staphylococcus aureus* has been shown to localize in a ring at the septum, the site of cell wall synthesis (Scheffers & Pinho, 2005). The PBPs of the coccoid bacterium *Streptococcus pneumoniae* either localize at the septum in duplicated equatorial rings that are the future division sites of the daughter cells, or are both septally and equatorially localized (Scheffers & Pinho, 2005). The authors postulated a model in which the PBPs are recruited via protein–protein interactions with division proteins that are located at the septum. Subsequently the multi-enzymic complex for cell wall synthesis is thought to be uncoupled from the cell division proteins and to remain localized through substrate recognition. Whether this mechanism is similar for both cocci and rods remains to be investigated.

Chemotaxis proteins

In chemotaxis, bacteria sense changes in their chemical environment and move away from repellents or towards more favourable conditions. In *B. subtilis*, chemotaxis proteins localize to the poles of the cell, as has been shown for the methyl-accepting chemotaxis proteins McpB and TlpA (Kirby *et al.*, 2000; Meile *et al.*, 2006). Information is transferred to the flagellar motors through phosphorylation of the soluble protein CheY, the concentration of which is influenced by external conditions. Localization of the phosphatase controls the spatial distribution of CheY-P in the cytosol. In *B. subtilis* the primary phosphatase involved is predicted to be located near the flagellar motors to minimize differences in the concentration of phosphorylated CheY proximal to each motor (Rao *et al.*, 2005). Polar localization of chemoreceptors in *B. subtilis* depends on a low attractant concentration and was proposed to be critical for signal amplification, whereas a high level of attractant resulted in a diffuse localization pattern, indicating that the proteins are mobile (Lamanna *et al.*, 2005). The authors propose a model in which chemoreceptor oligomers form an extended lattice that dissociates depending on the signal amplification required. In *E. coli* it has been shown that such chemoreceptors are first inserted into the membrane via the Sec machinery, and thus in a helical pattern, from which they are subsequently sorted to the cell poles (Shiomi *et al.*, 2006).

Sporulation proteins

There are several mechanisms by which proteins can reach their correct destination during sporulation, as was shown in *B. subtilis*. Proteins of the outer forespore membrane, which are expressed in the mother cell, probably insert randomly into the cytoplasmic membrane and subsequently diffuse to, and are captured in, the outer forespore membrane by interaction with other proteins (Rudner *et al.*, 2002). An example is the mother cell protein SpoIIIAH, which becomes tethered to the sporulation septum by interaction with the forespore protein SpoIIQ across the space between the mother cell and the forespore (Blaylock *et al.*, 2004). However, FtsY, involved in targeting

SRP-dependent proteins to the cytoplasmic membrane, becomes enriched in early sporulation septa, suggesting that some proteins are directly inserted at this location (Rubio *et al.*, 2005). Forespore-expressed membrane proteins initially localize to the septum, suggesting direct insertion at this site.

Cell-wall-located proteins

Proteins that are secreted and subsequently exposed on the cell surface or directly secreted into the external medium have to pass the negatively charged bacterial cell wall, which is composed of peptidoglycan with different types of molecules attached, such as (lipo)teichoic acids, teichuronic acids, polyphosphates and carbohydrates (Schaffer & Messner, 2005). Several factors have been shown to be important in retaining proteins without an identified cell-wall-binding domain in the cell wall by their influence on the charge of the cell wall (Hyyrylainen *et al.*, 2000; Calamita & Doyle, 2002; Nouaille *et al.*, 2004).

Covalently cell-wall-bound proteins

Covalently cell-wall-anchored surface proteins of Gram-positive bacteria possess the C-terminal cell wall sorting signal LPxTG (Navarre & Schneewind, 1999). During export of the protein, this sorting signal is cleaved between the Thr and Gly residues and the protein is covalently attached to peptidoglycan by a sortase. Although *B. subtilis* encodes two putative sortases, YhcS and YwpE, no sorted proteins have yet been identified. To immobilize proteins on the surface of this bacterium Nguyen & Schumann (2005) expressed sortase A from *L. monocytogenes* and a fusion of α -amylase to the C-terminal sorting domain of the fibronectin-binding protein B of *Staph. aureus*. The covalently bound fusion protein was shown by immunofluorescence microscopy to accumulate in patches in the cell wall, showing that cell wall anchoring takes place at specific sites. The authors suggest that the sortase might be located in the vicinity of the translocation sites to scan secreted proteins for the presence of a sorting signal. In contrast, the cell-wall-anchored M protein of *Strep. pyogenes* was shown by immunogold electron microscopy to be circumferentially distributed (Rosch & Caparon, 2004). Thus, it appears that the sortase is either randomly distributed or present at the ExPortal, from which the sorted protein might move away together with the newly synthesized cell wall. In the early 1960s it was already shown that the contact points between cells of a chain of *Strep. pyogenes* cells are the sites for cell wall synthesis and of appearance of anchored surface proteins (Cole & Hahn, 1962). Removal of surface proteins by proteolytic treatment and subsequent detection of newly synthesized proteins demonstrated that the M protein appeared at these contact points and extended slowly over the entire cell surface. Based on these results, Navarre & Schneewind (1999) suggested 27 years later that surface proteins, together with peptidoglycan, are incorporated at defined sites in order to coordinate protein sorting and cell wall synthesis. In light of

recent results, it could well be that these sites are connected to the ExPortal. Recently, we obtained results for *Lc. lactis* that support the first hypothesis of Navarre and Schneewind. In an exploratory study, we replaced the active-site domain of the sorted proteinase PrtP of *Lc. lactis* by the malaria parasite antigen protein MSA2 (Leenhouts *et al.*, 1999). Upon induced expression the fusion protein was primarily secreted and sorted near the septa of *Lc. lactis* cells, the sites of cell wall synthesis, after which it was distributed in time over the whole cell surface (unpublished results). Like many other streptococci *Lc. lactis* contains two sortase homologues, YlcC and YhhA (Comfort & Clubb, 2004). Fusion of GFP to either of the sortases resulted in fluorescent spots near the septum or the poles of the cell (unpublished results). Redistribution of cell-wall-bound proteins such as the proteinase PrtP could well be similar to that described in the model for ActA localization of *L. monocytogenes* (Rafelski & Theriot, 2006; see above).

Non-covalently cell-wall-bound proteins

A major group of proteins bound non-covalently to the cell wall is formed by the peptidoglycan hydrolases, which are, amongst other roles, involved in cell separation after division, cell wall turnover, autolysis and sporulation (Navarre & Schneewind, 1999; Scheffers & Pinho, 2005). Most peptidoglycan hydrolases contain a domain for non-covalent cell wall binding. The minor autolysins LytE and LytF of *B. subtilis* are DL-endopeptidases and play an important role in cell separation during cell division. The N-terminal cell-wall-binding domains of LytE and LytF contain three and five LysM domains, respectively. LysM domains consist of a stretch of around 45 amino acids that have been shown to bind peptidoglycan (Pfam01476; Steen *et al.*, 2003). Epitope-tagged derivatives of LytE and LytF localize at cell separation sites and cell poles in vegetative cells of *B. subtilis* (Yamamoto *et al.*, 2003). The major autolysin AcmA of *Lc. lactis*, which is involved in cell separation (Buist *et al.*, 1995), contains three LysM domains. Immunofluorescence analysis of a fusion protein containing these LysM domains revealed that the protein binds mainly at the polar regions of *Lc. lactis* and *B. subtilis* when added from the outside (Steen *et al.*, 2003). Identical patterns have been observed for cells producing such fusion proteins (unpublished results). Secretion of these proteins might take place at the site of binding, as was demonstrated for ATL of *Staph. aureus*, which is a bifunctional protein with an amidase and a glucosaminidase domain separated by three homologous repeated sequences. The two active-site domains become separated by proteolytic cleavage between the second and the third repeat and are each involved in cell separation. Immunoelectron microscopy showed that both proteins form a ring structure on the cell surface at the septal region for the next cell division site. A similar distribution of gold particles was observed on protoplasts, suggesting the presence of a receptor molecule such as lipoteichoic acid (Yamada *et al.*, 1996). The authors proposed that both parts of ATL are translocated at the cell division site, followed by

binding to a receptor molecule. The non-covalently cell-wall-bound lytic transglycosylase IsaA of *Staph. aureus* is also mainly present in the septal region of dividing cells, and thus is possibly secreted at this site (Sakata *et al.*, 2005). It therefore appears to be a common characteristic for many non-covalently cell-wall-bound proteins that they are secreted at the site of cell wall binding, where their biological activity may be needed. However, this is not true for all of these proteins, since a FLAG-tagged LytC protein of *B. subtilis*, which contains an N-terminal cell-wall-binding domain, is present on the whole cell surface while its secretion most likely takes place at the poles/septum or along the spiral (Yamamoto *et al.*, 2003).

Mechanisms controlling localized protein secretion

The location of secretion machineries and their substrates raises the question as to how and why these systems are maintained at these sites.

Cytoskeletons and cell wall synthesis

Cell wall synthesis in rod-shaped bacteria is thought to occur via two modes, one responsible for formation of the division septum and one for cell elongation (Scheffers & Pinho, 2005). Coccoid bacteria possess only the first mode, in which the tubulin homologue FtsZ recruits cell-division-specific wall synthesis enzymes such as the PBPs at the septum via the formation of a so-called Z-ring.

Using a fluorescent derivative of vancomycin as a probe for nascent peptidoglycan synthesis, cell wall synthesis in the rod-shaped bacterium *B. subtilis* was shown to occur in a helical pattern that appears similar to that found for protein secretion (see above). This process was shown to be governed by actin-like cables of Mbl (Daniel & Errington, 2003), although a recent report in which the helical staining was also observed in the absence of Mbl calls this into question (Tianan *et al.*, 2006). With the same approach the coccoid bacterium *Staph. aureus* has been shown to synthesize new cell wall material at the cell division sites in the form of a flat disc that is subsequently cleaved and remodelled to produce the new hemispherical poles of the daughter cells (Pinho & Errington 2003).

B. subtilis contains three actin-like proteins that are involved in cell shape determination by exerting spatial control over the cell-wall-synthesizing machinery. MreB and Mbl each form a distinct filamentous helical structure close to the cell surface (Jones *et al.*, 2001). Based on comparison of the localization patterns for cell wall synthesis and protein secretion one could therefore speculate that Sec-mediated protein secretion takes place near the sites of cell wall synthesis. The helical structures are not identical, however, since in *B. subtilis*, SecA and SecY retain their helical localization in the absence of MreB or Mbl (Campo *et al.*, 2004), although the presence of only one of these proteins might be sufficient. In addition, recent localization experiments of ActA-RFP and vancomycin in

L. monocytogenes also suggest that the internal scaffold directing helical cell wall growth may be distinct from the location of the Sec apparatus (Rafelski & Theriot, 2006). In the Gram-negative bacterium *E. coli* the helical organization of the Sec machinery also appeared distinct from the MreB coil (Shiomi *et al.*, 2006). Interestingly, the localization of various PBPs also did not depend on MreB or Mbl (Scheffers *et al.*, 2004). LytE localization in *B. subtilis* was recently proposed to be directed by the third actin homologue MreBH (Scheffers & Pinho, 2005). LytE-GFP was observed at the cell poles, at the cell division site and at discrete sites in the cylindrical part of cells. Lateral wall localization of LytE was abolished in an MreBH mutant, while septal localization was dependent on division proteins. It was proposed that LytE transiently associates with MreBH in the cytoplasm and is recruited to specific sites in the cytoplasmic membrane, where it is secreted and accumulates extracellularly. It will be interesting to unravel whether other proteins are also secreted in an MreBH-dependent fashion.

The model proposed by Rafelski & Theriot (2006), namely that the Sec translocons are fixed in space while the cell wall grows around them, could provide a mechanism for redistribution of cell surface proteins as well as for their transport over the thick cell wall. Cell wall synthesis could act as driving force to push a large protein that has to be translocated outwards into the external medium. As proteins up to 25 kDa can cross the cell wall by diffusion (Demchick & Koch, 1996), this could be the reason why dedicated secretion machineries for small proteins such as pheromones and bacteriocins do not seem to be localized (Gajic *et al.*, 2003). A possible periplasmic space, the presence of which was evidenced for *B. subtilis*, would provide room to move protein folding away from the highly negatively charged cell wall polymers (Merchante *et al.*, 1995; Matias & Beveridge, 2005). Diffusion through this space to the most permeable region of the cell wall could be a general way to release smaller proteins. Determining how Gram-positive bacteria transfer large secreted proteins across the cell wall is a challenging question for future research.

Lipid domains

Lipid domains have been detected in bacterial membranes, and were shown to dissipate upon inhibition of protein synthesis (Fishov & Woldringh, 1999; Vanounou *et al.*, 2003). These domains appear to be connected with nucleoid partitioning and division. They were proposed to be formed by specific protein-phospholipid subdomains connected to DNA and are ascribed to the presence of so-called transertion structures, created by the process of coupled transcription, translation and insertion of nascent membrane- and exported proteins (Binenbaum *et al.*, 1999; Fishov & Woldringh, 1999). If such transertion structures exist, they should contain Sec proteins, since the Sec machinery constitutes the main protein translocation machinery. Indeed, spiral-like localization of SecA in *B. subtilis* depends on the growth phase of the cells, on active

transcription/translation and on the presence of negatively charged phospholipids (Campo *et al.*, 2004). The nature and localization of membrane subdomains in *B. subtilis* is currently not clear, however. Cardiolipin-rich domains are present in the septal region and at the poles (Kawai *et al.*, 2004), while phosphatidylethanolamine also appeared to be preferentially localized in the septal region (Nishibori *et al.*, 2005). In fact, most enzymes involved in lipid synthesis appear to be localized at the septum (Nishibori *et al.*, 2005).

Conclusions and perspectives

From the results summarized in this review it is clear that protein secretion takes place at various locations depending on the type of bacterium and the type of secretion machinery. The data also suggest that in all Gram-positives investigated thus far the general Sec machinery is located near the site(s) where cell wall synthesis takes place. Dedicated secretion machineries for small proteins such as pheromones and bacteriocins do not seem to be localized. How components of the various secretion machineries are maintained at specific places and whether protein-protein interactions, interaction with the cell wall synthesis machinery or actin-like proteins and lipid rafts are involved remains to be investigated.

Although the rigid bacterial cell wall is designed to withstand turgor pressure it also must be porous to allow the passage of proteins and peptides. It could be that the cell wall is heterogeneous in structure and more open at the site(s) of protein secretion. Restriction of protein cell wall passage to only a limited number of sites, close to where these proteins are translocated over the cytoplasmic membrane, would result in a minimal required porosity of the cell wall. This would suggest a well-balanced control of protein secretion and cell wall synthesis. Many more studies on the localization of secretome and cell wall components are required to elucidate the actual mechanism(s).

A very interesting puzzle is the surface localization of the covalently cell-wall-bound M protein of *Strep. pyogenes*. Although a single microdomain for protein secretion was identified in this bacterium, the M protein is present on the whole cell surface. How the distribution of this protein is directed remains to be elucidated.

Simultaneous *in vivo* time-lapse analysis of multiple components of secretion machineries, their substrates and other components involved will have to be performed to generate more insights into the questions raised. The current rapid development of novel fluorescence techniques will be of great importance to make such studies feasible (Giepmans *et al.*, 2006; Lorenz *et al.*, 2006).

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